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**Mustard Contaminated Soil: Understanding the  
mechanisms and parameters governing contaminant  
behaviour and biodegradation**

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### Executive summary

Mustard is a highly toxic chemical warfare agent which has contaminated soil at certain old military sites in the UK and across the globe. When in soil, it reacts with water to form hydrolysis products, but this process also leads to the formation of dimers and polymers especially at high concentrations. Its presence in soil for over 60 years is indicative of its toxicity and lack of accessibility to microorganisms (bioavailability). To achieve enhanced remediation of the soil through biological processes it is necessary to understand the complex chemistry in combination with the effects upon the microbiology and health of the soil. This is the first reported study aimed at investigating:

- the impact of buried mustard agent and its hydrolysis products on exposed soil microbial communities and;
- the subsequent biodegradation of the contaminant.

In soils contaminated with mustard, higher mustard concentrations led to a lower pH (low oxygen and nitrogen concentrations were also observed), which inhibited contaminant biodegradation in the contaminated sites. pH values, specifically  $>6$ , enhanced biodegradation of the mustard hydrolysis product, thiodiglycol (TDG), in microcosms of soils free from mustard contamination. Significantly higher sulphate and chloride concentrations were detected in mustard contaminated soil, compared to uncontaminated soil, due to the mineralisation of mustard agent.

The soil microbial community was not affected by the presence of TDG alone, but in soil contaminated with mustard agent it was depleted compared to the large number of species in the clean soil control sample. It was not possible to isolate a single organism capable of degrading TDG from uncontaminated soils. However a consortium of three bacteria, *Rhizobium* sp, *Achromobacter* sp, and *Stenotrophomonas* sp could biodegrade the compound. One single bacterial strain, identified as a *Burkholderia* sp., was isolated from contaminated soil and shown to be capable of utilising TDG as a sole carbon source for growth. This is good evidence that evolution of TDG-degrading capability had occurred in the contaminated soils.

Attempts to isolate degrading microorganisms for 1,4 dithiane and 1,4 thioxane failed, suggesting that constraints such as low solubility in water, and hence low bioavailability, play an important part in the fate of these compounds in soil.

It is anticipated that minor intervention, principally through incorporation of lime and a high nitrogen fertilizer, at a mustard contaminated site would lead to accelerated destruction of TDG and, possibly, of the residual mustard. Additional intrusive intervention to aerate the soil, perhaps through rotovation, may also assist in promoting biodegradation.

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## 1 Introduction

Sulphur mustard (Bis(2-chloroethyl) sulphide (H)) is a highly toxic chemical warfare agent which has contaminated soil at certain old military sites in the UK and across the globe. At room temperature pure mustard is an odourless and colourless oily liquid [1](Malhotra, et al., 1999). The name comes from impure weapons-grade material, which has an odour similar to that of mustard, garlic or horseradish. When in soil it reacts with water to form hydrolysis products, principally thiodiglycol (TDG) and hydrochloric acid, but this process also leads to dimerisation and polymerisation especially at high concentrations. The toxicity of mustard is due to the fact that it is a strong alkylating agent and damages cells, particularly the DNA, so it is toxic to most organisms. However, because of its low solubility in water and rapid rate of hydrolysis to TDG, it is not bio-available and is not likely to come into direct contact with microorganisms which instead metabolise the water soluble breakdown product. In soil the hydrolysis of mustard is dependent upon environmental conditions and mustard agent can persist for many decades [2], indicating that the hydrolysis process can, in some conditions, be very slow. The biodegradation of TDG derived from hydrolysis of mustard has previously been demonstrated [3], [4] and [5]. However, TDG degradation in soils, the impact of TDG on exposed soil microbial communities and, particularly, the microbial functional groups that may play a critical role in the degradation of TDG, have received relatively little attention [6] and [7].

To achieve enhanced remediation of mustard contaminated soil through biological processes, it is necessary to understand the complex chemistry of the system in combination with the impact of mustard and its breakdown products upon the microbiological and health of the soil. This is the first reported study aimed at investigating:

- the impact of mustard agent on exposed soil microbial communities and;
- the biodegradation of the mustard agent hydrolysis products.

## 2 Background

TDG is the primary hydrolysis product of sulphur mustard but is also a key precursor for its chemical synthesis. One method developed to destroy the remaining stockpiles of mustard used a hydrolytic approach followed by biodegradation of the thiodiglycol produced. The biodegradation step is necessary because TDG is classified as a Schedule Two Compound (i.e. a key precursor) in the Chemical Weapon Convention (CWC) because it can be converted to sulphur mustard by the addition of chlorinating agents such as concentrated hydrochloric acid. The hydrolysis of mustard followed by the biodegradation of TDG has been successfully employed for destroying stockpiles in the USA [3], [4] and [5].

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Once mustard has been hydrolysed to TDG its toxicity decreases significantly. The LD50 (Rat, oral) of TDG is 6610 mg/kg [2] compared to 2.4 mg/kg for mustard [8].

TDG bio-transformation in soil has been studied [9] using soil suspensions, and although it has been demonstrated that TDG can be oxidised to thiodiglycolic acid (TDGA), information on the factors affecting soil biodegradation of TDG is limited [10]. What is clear, however, is that mustard and TDG can continue to persist in the environment, in some cases for more than 60 years. One mechanism that has been suggested to contribute to the persistence of TDG is that it reacts with mustard and/or intermediates during the hydrolysis process to form toxic and persistent TDG aggregates [2]. An outer layer of TDG aggregate probably shields the bulk of mustard from further dissolution so contributing to the persistence of mustard in soil. Because TDG would be expected to migrate away from a site, disperse and biodegrade, its presence indicates that it is being continuously produced by reaction of a low solubility source, which is probably the protected mustard aggregate. It has been reported that the hydrolysis reaction of mustard may be reversible [2]. Thus it seems evident that one mechanism to aid the clean up of soils contaminated with mustard agent and TDG is to stimulate rates of biodegradation to remove the breakdown products from the system and prevent the back reaction. Understanding the constraints to biodegradation and the microbiology of the contaminated sites is essential for such remediation.

### 3 Aims

3.1 The aim of the research presented here are as follows:

- to understand the microbiology of mustard contaminated soils (in terms of activity and diversity) and how this is related to soil properties and contaminant chemistry;
- to understand the biodegradation of mustard and its breakdown products in contaminated soils, including governing parameters and constraints;
- to develop and assess methods to overcome the constraints to bioremediation;
- to understand how a range of physical and chemical soil conditions influence the long term fate, availability and toxicity of mustard agent; and
- to isolate and characterize bacteria that are tolerant to, and capable of biodegrading, mustard or its breakdown products.



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4 Sampling

4.1 Sampling

No sampling was specifically carried out to support this work. The samples were drawn from three prior investigations at a single contaminated site. All of the samples were collected in the same general area of the site, however they were separated temporally by one to two years.

The purpose of this report is to compare soil chemistry and microbiology between contaminated and uncontaminated soils therefore a very limited description of the sampling exercises is given. The sampling exercises are as summarised below in table 1.

Sampling Exercise	Contaminated Samples	Uncontaminated Samples	Depth of Samples	Remarks
A	The mean of 5 surface samples.  A further single sample was taken at a location known as site C.	Details unavailable.	15 to 30 cm	Contaminated surface samples were taken at locations where a Chemical Agent Monitor (CAM) alarmed.
B	The mean of 17 samples taken from 6 boreholes.	The mean of 14 samples taken from 5 boreholes.	Every 30 cm between 30 and 120 cm.	Investigation by the Army. Bore holes only.
C	The mean of 7 samples from each borehole. Three boreholes BHD, BHF and BHM.	The mean of 7 samples taken at borehole BHO.	Every 30 cm between 30 and 210 cm	Investigation by Dstl. Bore holes and vapour tubes.

Table 1- Data Sets recovered from Sampling Exercises A, B and C

For the remainder of this document each sampling exercise will be referred to as Exercise A, B and C.

## 5 Investigation and Results

### 5.1 Microbiology and chemistry of sulphur mustard contaminated sites

#### 5.1.1 Relationship between soil pH and mustard contamination

The pH of mustard agent contaminated soil should drop as the acidic breakdown products TDGA and HCl increase in concentration. As a simple first test of this expectation, 5 representative samples were taken from 5 locations within the contaminated site [11] and the pH measured in each case. The results for each location were averaged and plotted in Figure 1. The results show a correlation between mustard agent concentration and pH with a negative linear correlation ( $r = -0.933$ ,  $p = .020$ ) between pH 8.5 and 4.5 and up to 19 ppm of mustard.

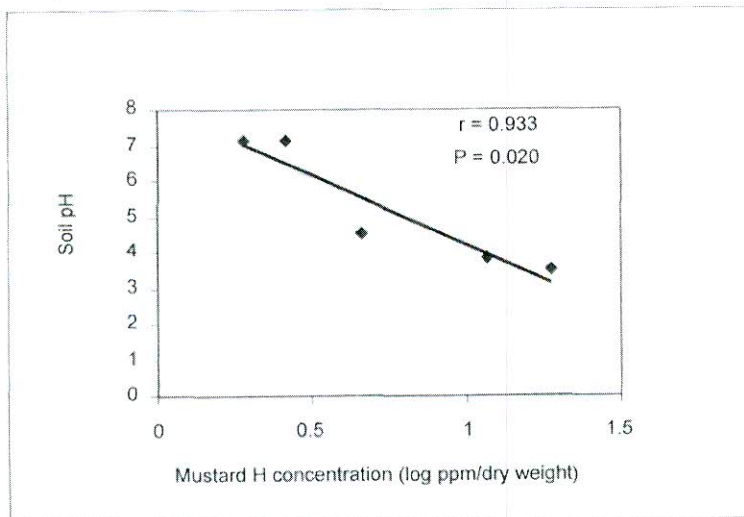


Figure 1 - Effect of sulphur mustard concentration on soil pH.

As a further test of the dependence of pH on the concentration of mustard, batches of samples were taken from sampling Exercises A, B and C. The results of pH measurements are summarized in Figure 2, (a) to (c). Error bars in each case are the  $\pm$  standard errors of the mean.

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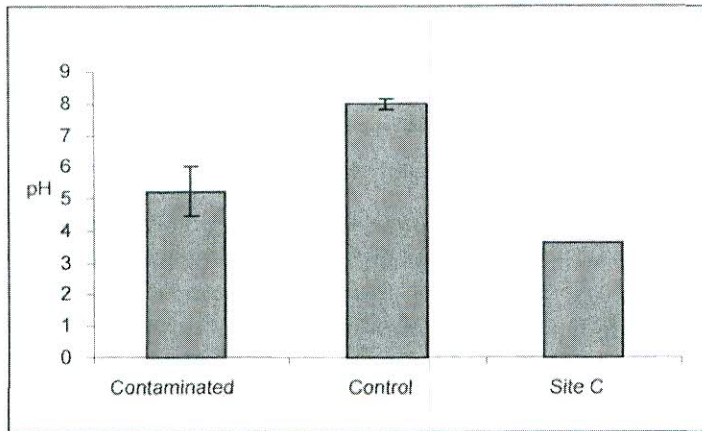


Figure 2 (a) - Comparison of pH for contaminated and clean soils collected during Exercise A.

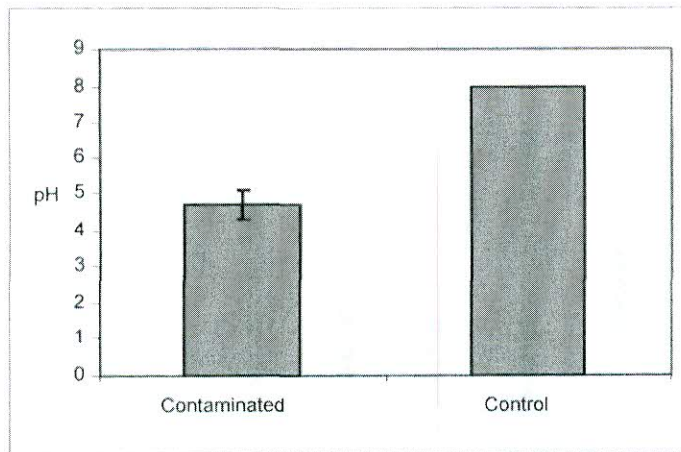


Figure 2 (b) - Comparison of pH for contaminated and clean soils collected during Exercise B.

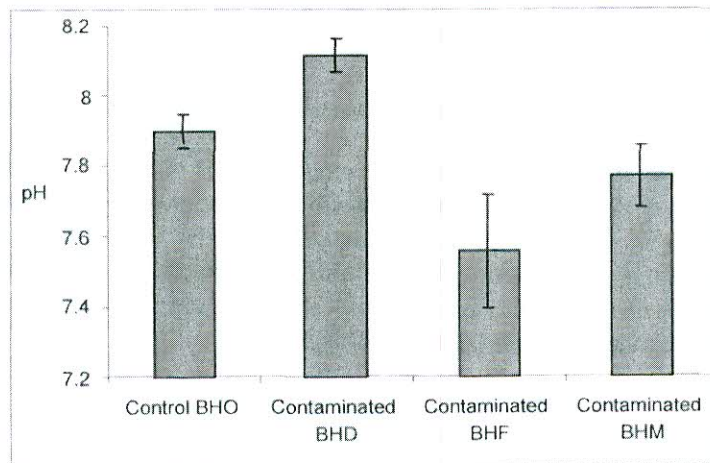


Figure 2 (c) - Comparison of pH for contaminated and clean soils collected during Exercise C.

The results confirm the findings in Figure 1 and show the reduced pH values at the contaminated sites compared to clean soil in the control. Figure 2 (c) demonstrates the

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variation of pH at different levels of contamination within a contaminated site which almost certainly reflects the different concentrations of mustard. The results for BHD appear anomalous, however, in that the pH is higher than for the control. There is no obvious explanation for this unless BHD was in clean soil and the observed difference in pH is due to natural variation across the site.

In order to investigate the effects of pH on bio-degradation of TDG, clean soil from Wytham was contaminated with TDG and amended with phosphoric acid solution to the pH values between 3.5 and 9.5. The TDG concentrations were measured in each case at daily intervals and, although degradation is shown at all pH values, the amount removed is less under strongly acid conditions and there is a clear optimum between pH 7.5 and pH 8.5 (see Figure 3) although it should be noted that any pH above ~6 gives rise to a significantly enhanced rate of degradation. This implies that biodegradation of TDG in soil is inhibited at low values of pH.

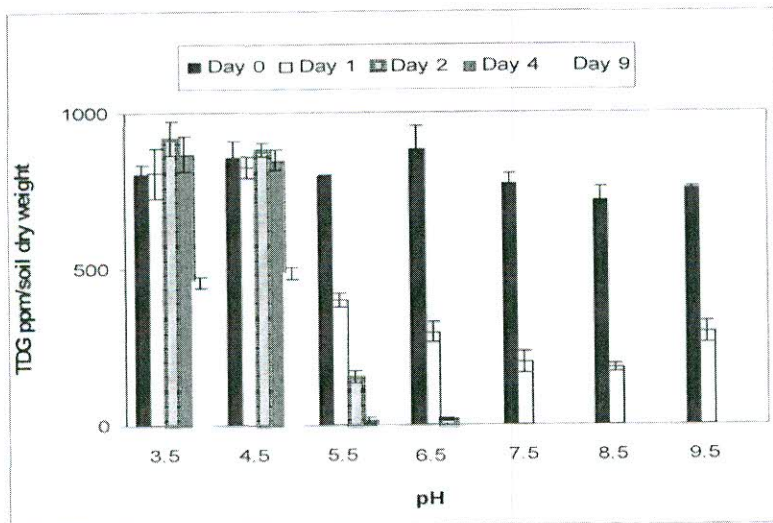


Figure 3 - The effect of soil pH on degradation of TDG in Wytham soil

### 5.1.2 Concentrations of characteristic chemical species in mustard contaminated soil

It is anticipated that the concentrations of certain characteristic chemical species will change as biodegradation proceeds. In particular, chloride and sulphate levels are expected to increase as the heteroatoms in the mustard molecule are released, and in the case of sulphur oxidized, and the chemical and biological breakdown processes proceed until complete mineralisation is achieved. Figures 4 (a) – (b) and 5 (a) – (c) summarise analysis carried out to characterise and quantify this process.

Figures 4 (a)-(b) show the variation in concentrations of TDG and its primary biodegradation product TDGA in samples from Sampling Exercises B and C (unfortunately there was insufficient sample material available to carry out TDG/TDGA analysis for Exercise A material). Both of these compounds are very soluble in water and would be expected to leach out of the soil at a steady rate as well as being destroyed by biodegradation. These factors probably account for the variation in concentrations

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observed at the various contaminated sites. The presence of TDG/TDGA implies the presence of mustard aggregates.

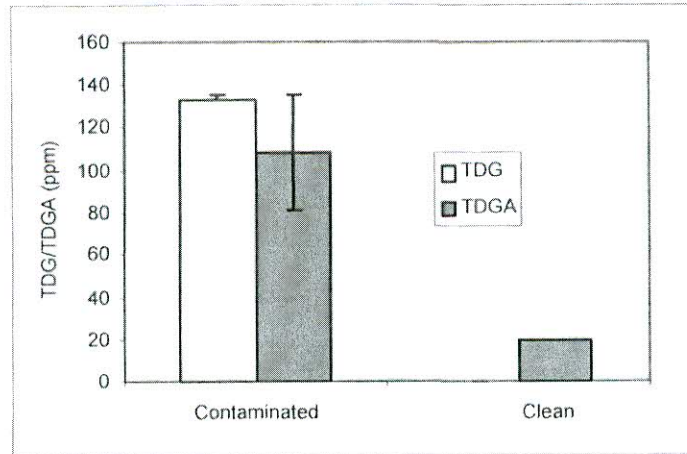


Figure 4 (a) - TDG and TDGA concentrations in contaminated soils collected during Exercise B

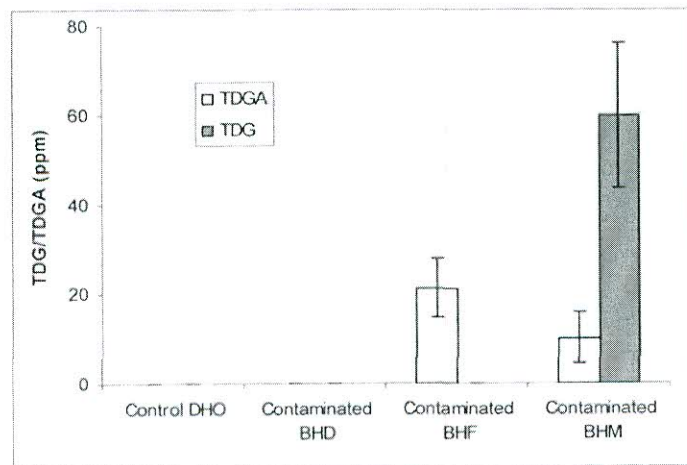


Figure 4 (b) - TDG and TDGA concentrations in contaminated soils collected during Exercise C

Figure 5 records the measured concentrations of chloride and sulphate. Both ions are present in all samples. The chloride and sulphate concentrations in the control samples represent the background concentrations in the local soil. In every case, except borehole F on from Exercise C, chloride concentrations are significantly lower than sulphate concentrations despite the fact that there are two atoms of chlorine in each molecule of mustard compared to one of sulphur. In addition, chloride is a primary hydrolysis product of mustard whereas sulphate is one of the final products at the end of the chemical and biological degradation process. This observed difference in concentration is almost certainly due to the high solubility of chloride ions which consequently leaches away rapidly in the ground water compared to the relatively insoluble sulphur salts.

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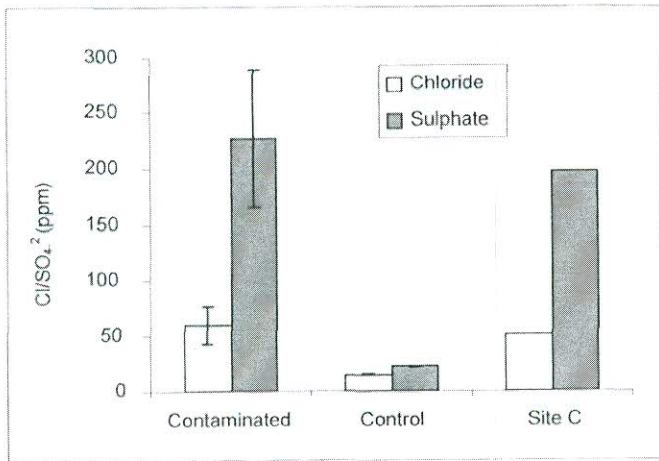


Figure 5 (a) – Chloride and sulphate concentrations in soils collected during Exercise A.

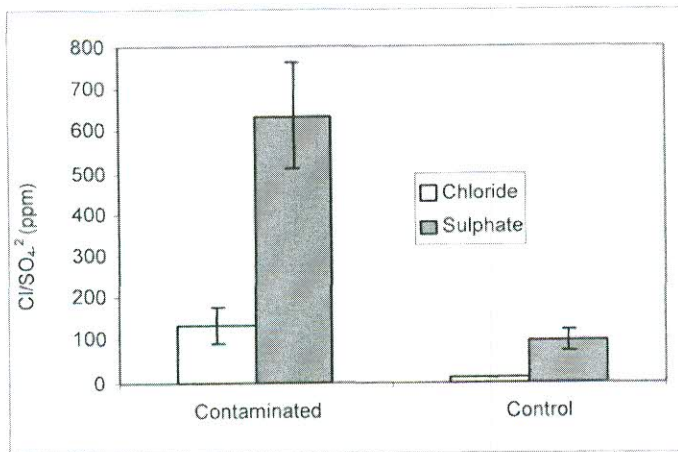


Figure 5 (b) – Chloride and sulphate concentrations in soils collected during Exercise B.

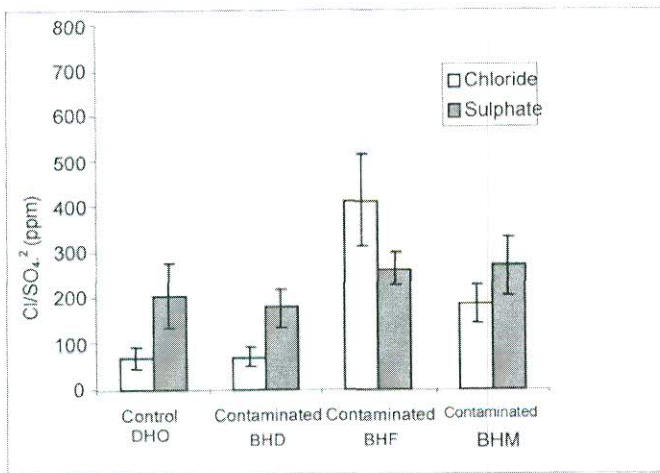


Figure 5 (c) – Chloride and sulphate concentrations in soils collected during Exercise C.

5.2 Overcoming the constraints to bioremediation

5.2.1 Limitations to the degradation of TDG due to lack of nitrogen

Nitrogen is a key element in the growth of microorganisms and its absence can seriously constrain the proliferation of microbial colonies. Nitrate concentrations were measured in both contaminated and clean soils from each exercise and the results are recorded in Figure 6 (a) – (c).

The results show that nitrate levels in the contaminated samples are much lower than the background levels. The most likely explanation for this observation is that soil nitrate is being depleted by the soil microorganisms on the contaminated soils where it is the main, or only, source of nitrogen. If this is so, then it is likely that the lack of bio-available nitrogen is inhibiting the growth of soil microorganisms in the contaminated soil and consequently limiting the continued biodegradation of TDG.

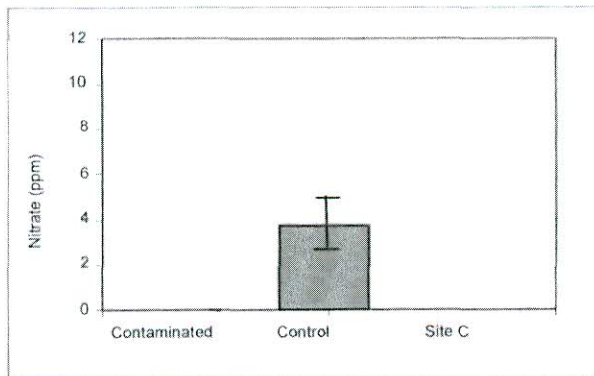


Figure 6 (a) Soil nitrate concentrations in soils from Exercise A

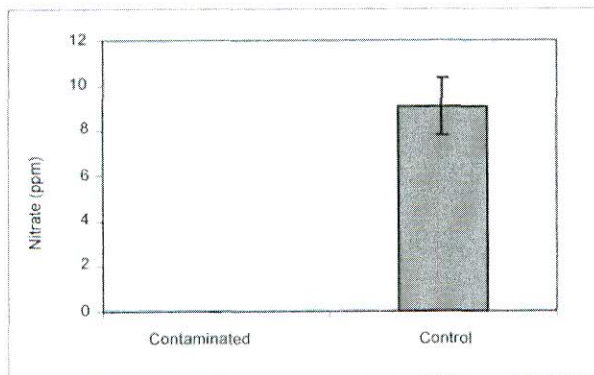


Figure 6 (b) Soil nitrate concentrations in soils from Exercise B

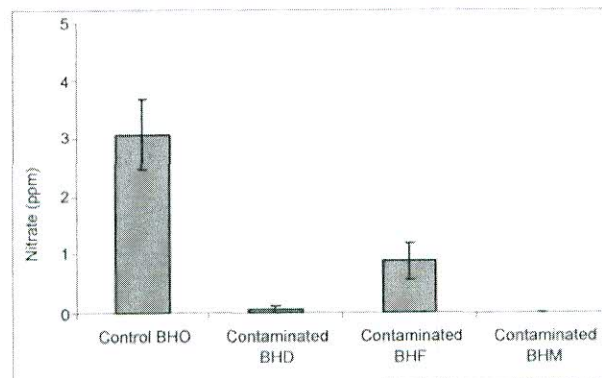


Figure 6 (c) Soil nitrate concentrations in soils from Exercise C

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To establish whether the low nitrate levels in the contaminated soil are inhibiting TDG degradation, the following experiment was carried out. Soil samples were taken from three contaminated borehole samples, (a) BHF, (b) BHD, and (c) BHM collected during Exercise C. Five grams of soil (dry weight) from each of the samples was mixed with 15 ml of TDG and nitrate solution. The resultant soil slurry had a final TDG concentration of 1.8g/l and nitrate concentration of 5mM. The soil slurry samples were then incubated at 25°C in a shaking incubator at the rate of 100rpm. The incubated samples were analysed for TDG at intervals and the results plotted in Figures 7(a)–(c). The results show in each case that, although degradation of TDG took place in the contaminated soils without extra nitrate, the process was always faster when this essential nutrient was added. It is therefore likely that soil nitrate is not the sole nitrogen source available to the microorganisms but it is not readily available so that the rate of degradation of TDG is inhibited to some extent by a lack of available nitrate on mustard contaminated sites.

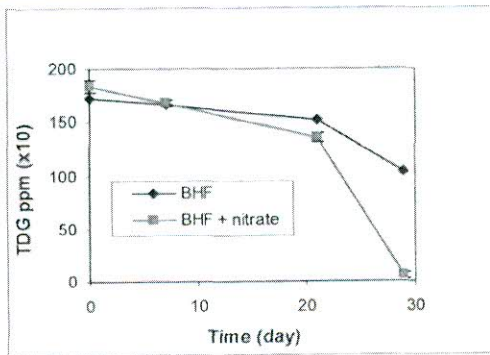


Figure 7 (a) - Effect of nitrate on TDG degradation in contaminated soils from borehole F.

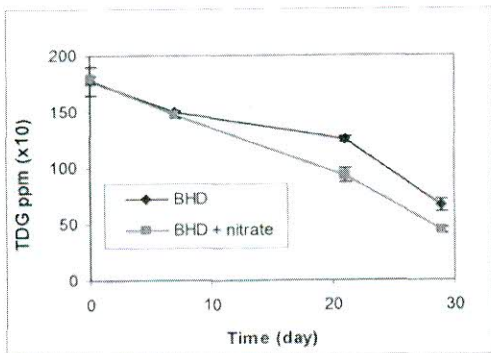


Figure 7 (b) - Effect of nitrate on TDG degradation in contaminated soils from borehole D.

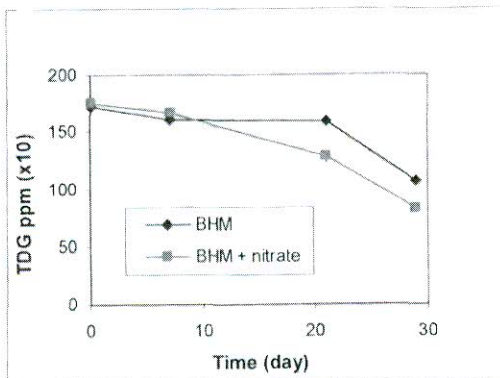


Figure 7 (c) - Effect of nitrate on TDG degradation in contaminated soils from borehole M.



5.2.2 Limitations to the degradation of TDG due to lack of oxygen

In order to investigate limitations to the degradation of TDG due to lack of oxygen, clean and contaminated soils were spiked with approximately 1000 ppm of TDG and incubated under aerobic (supplied air) and anaerobic (supplied pure nitrogen gas) conditions. In the case of heavily contaminated soil, incubation was only conducted under aerobic conditions because of sample limitations. All samples were incubated at 25°C.

The results of these experiments are displayed in Figure 8 and indicate that aeration gave rise to some enhancement of biodegradation of TDG in mustard contaminated soil. It was also observed that the biodegradation of TDG was faster in contaminated soil than in clean soil (although no degradation was observed in heavily contaminated soil). The enhanced biodegradation effect in contaminated soil was probably due to bacterial species which had adapted to degrade TDG. This proposition has been confirmed by isolating a bacterial species which can use TDG as sole carbon source from the contaminated site (see section 5.3). Aeration, together with optimizing the pH of the soil (see section 5.1.1) could be a simple approach to an enhancement of the biodegradation of TDG in contaminated soils, although it is clear that optimisation of pH has the most significant impact.

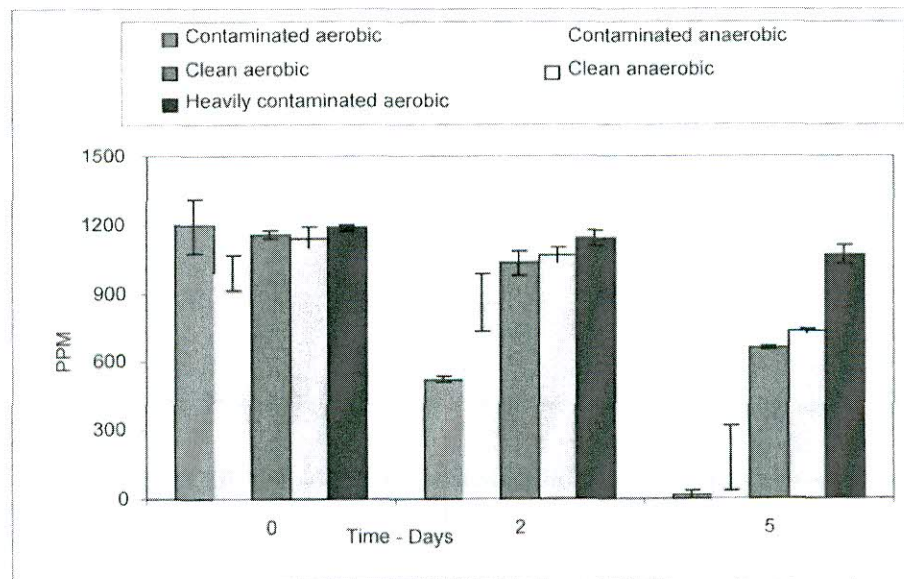


Figure 8 - Effect of aeration on TDG degradation in clean and contaminated soils

5.3 Investigation of the effects of mustard contamination on populations of soil microorganisms

Experiments were carried out to investigate the effects of contamination by mustard and its breakdown products on populations of soil microorganisms. Two separate methods were used for the investigation as follows.

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Uncontaminated soils were used from Reading, Sourhope and Wytham. In the first method used, soil samples were incubated for 5 days and used to inoculate Biolog Eco plates which were then further incubated at 16°C. Colour development in each well of the Biolog plates was then measured at different time points and used for statistical analysis. Figure 9 which also shows the results of the Principle Component Analysis (PCA).

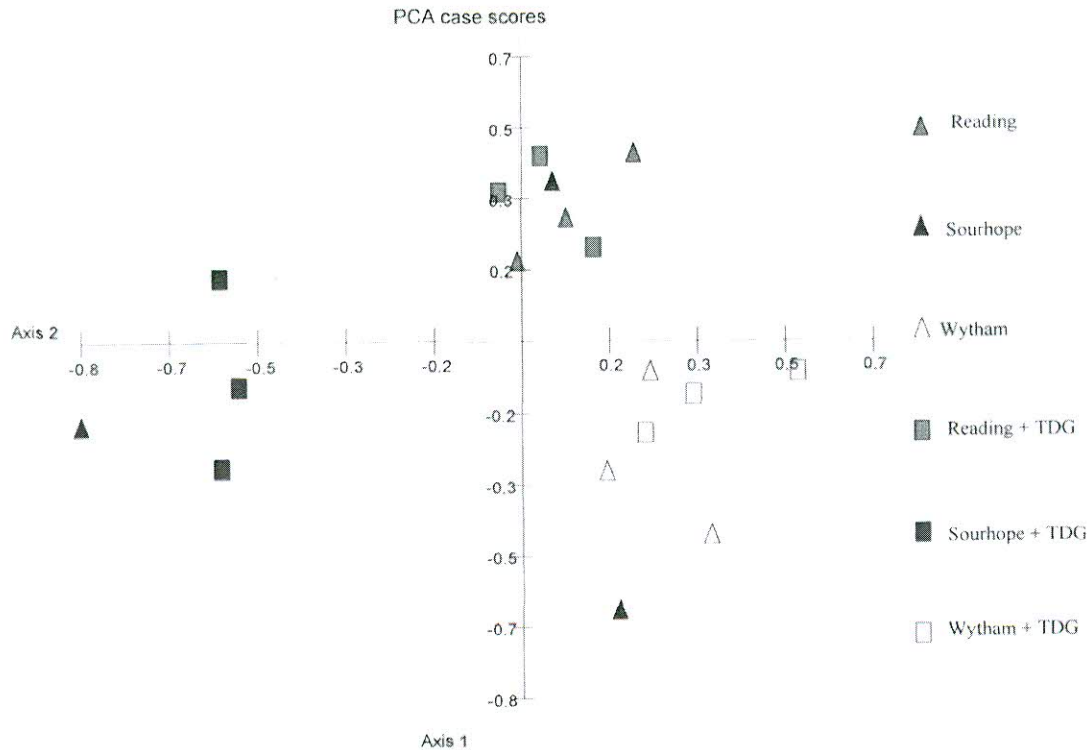


Figure 9 - Principle Component Analysis for microbial Community Level Physiological Profile

When measuring only two variables, such as microbial activity associated with different carbon source types in different types of soil, it is easy to plot the data and visually assess the correlation between the two factors. However, in an experiment such as the Biolog plate experiment described above, there are more factors involved, such as the presence and absence of TDG, soil type, carbon source utilization and different incubation time. Therefore, it becomes impossible to make a visual inspection of the relationship between carbon source utilization (microbial physiological profile) and addition of TDG in such a multi-dimensional matrix. One way to make sense of this data is to reduce its dimensionality and Principle Component Analysis is one of the methods for doing this. After the analysis, correlations between samples or conditions can readily be visualized. In the case of above experiment, the same type of soil produced similar scores. That is to say the results tended to cluster together (although Sourhope soils exhibited some scattering between repeats). Different soil types, however, behaved differently from each other and the results tended to cluster according to the soil type. This is normal as different soils have different microbial communities. However, within batches of the

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same soil, with and without the addition of TDG, the scores were also very similar (the results clustered together), indicating that the microbial physiological profile, in terms of carbon source utilization, was not changed by the addition of TDG to the soil.

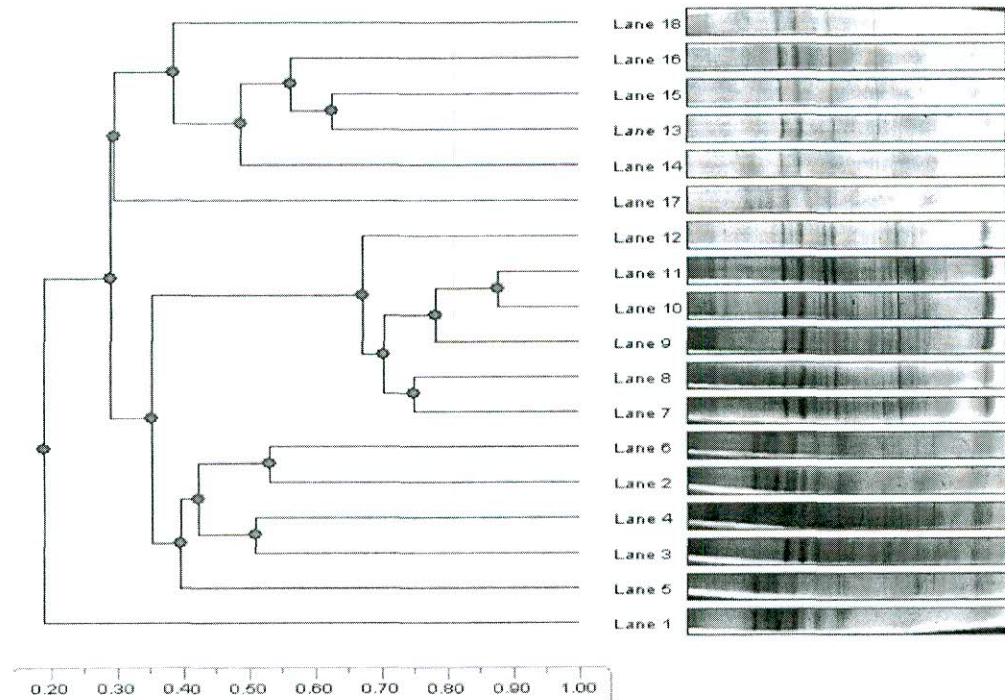


Figure 10 - Microbial community structure profiles analysis

Soil samples taken on day 5 during incubation were also used for Deoxyribonucleic Acid (DNA) excretion, 16S rRNA (ribonucleic acid) gene amplification by PCR (polymerase chain reaction) using a bacterial general primer. PCR products were then used for denaturing gel gradient electrophoresis (DGGE), or molecular “fingerprinting”. Cluster analysis of microbial community profiles was based on the presence or absence of bands (each band represents a microbial species) in the DGGE profiles (band patterns). The results are shown in Figure 10 where lanes 1 to 3 are for Reading soil, 4 to 6 for Reading soil + TDG, 7 to 9 for Sourhope soil, 10-12 for Sourhope soil + TDG, 13-15 for Wytham soil and 16-18 for Wytham soil + TDG. Again, the soil type was the dominant factor affecting the clustering with results for the same type of soil tending to be grouped together. No clear grouping is discernable when TDG was added to soils (no significant change in band pattern on addition of TDG), indicating that the addition of TDG had neither suppressed nor induced specific bacterial growth. Therefore, it can be concluded that the biodegradation of TDG in soils never previously exposed to the contaminant was due to the indigenous microbial community which was not unduly affected by the presence of TDG.

#### 5.4 Isolation and characterisation of tolerant and degradative bacteria

Tests were carried out to determine the frequency of bacterial functional groups in the soil based on average well development using the Biolog EcoPlate method. Samples from the contaminated site were used for Community Level Physiological Profile (CLPP) analysis by Biolog EcoPlate to test the diversity of microbial functional groups when using different carbon sources. The colour reading value in each well of the plate was subtracted from the value of the control well. The values produced were used to calculate the Average Well Colour Development (AWCD).

The results show that the Microbial CLPP was lower in contaminated soils (Figure 11), especially the heavily contaminated soil which showed far fewer diverse microbial functional groups, compared to uncontaminated soils. This means that the microbial community in the contaminated soil was impoverished compared to the large number of species in the clean soil control sample.

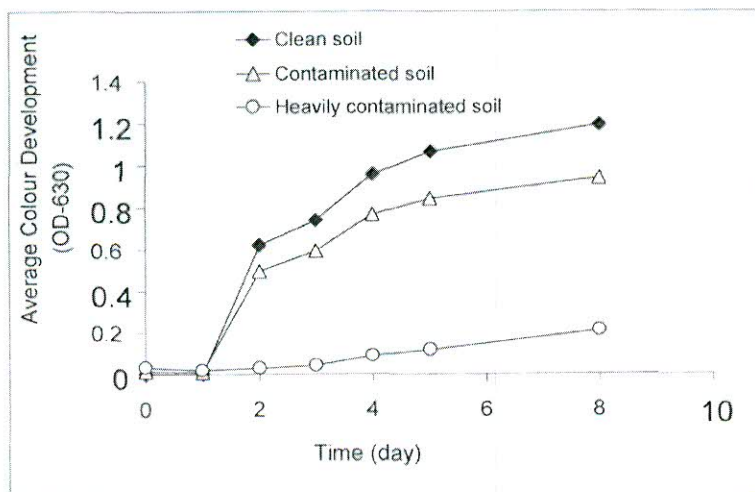


Figure 11 - Microbial Community Level Physiological Profile (CLPP) in soils from the sites

Experiments were carried out in an attempt to isolate novel bacterial strains capable of degrading mustard from the contaminated soil. A minimum concentration of salts including 2.0g /l TDG was used to culture the bacteria in 250-ml flasks containing 50 ml of growth medium. The flasks were incubated in a shaking incubator at a rate of 100 rpm at 25°C.

One strain, identified by 16S rRNA gene sequencing as a *Burkholderia* sp., was shown to be capable of utilising TDG as a sole carbon source for growth (Figure 12). It was also demonstrated that this species could grow on mustard hydrolysate, derived from the contents of old chemical munitions, which it utilised as a sole carbon source (Figure 13).

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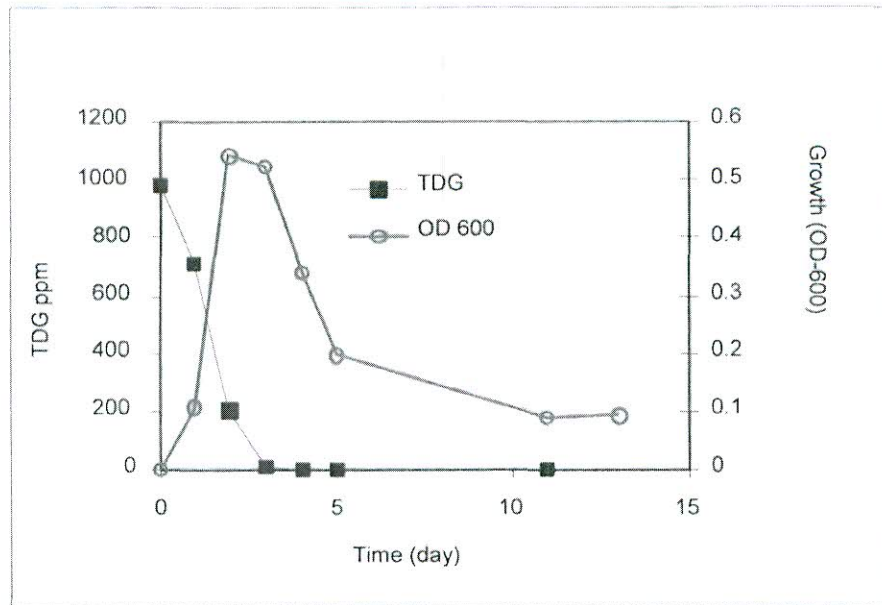


Figure 12 - TDG utilization and growth by bacterial strain *Burkholderia* sp isolated from contaminated soil

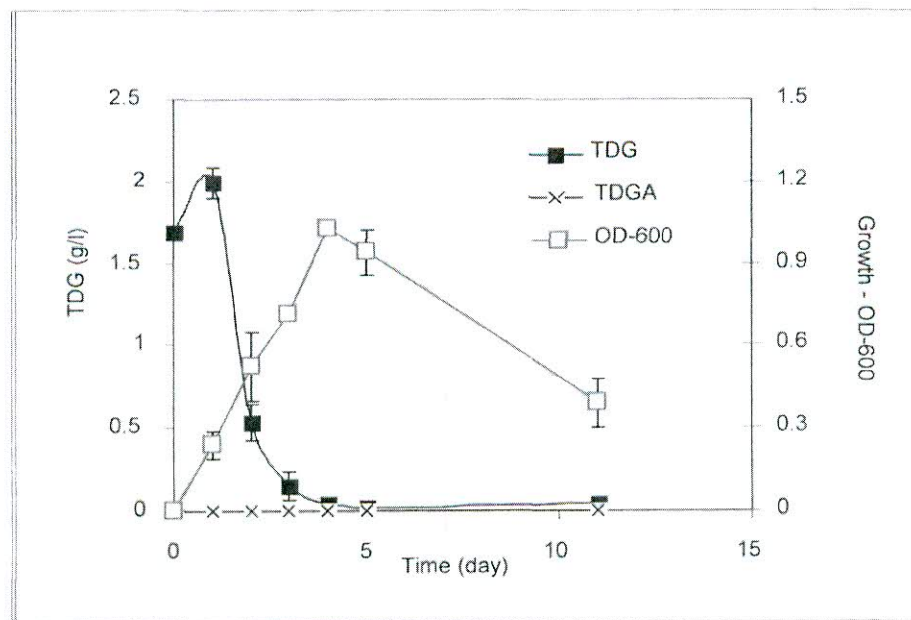


Figure 13 - Bacterial strain *Burkholderia* sp degrading TDG produced by hydrolyzing mustard in historical mustard bomb

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To test whether bacteria in all soils are capable of biodegrading TDG, three very different soil types were taken from across the UK and 1000 ppm TDG was added to each in a microcosm. These microcosms were used to culture the bacteria that could degrade TDG. It is significant that it was not possible to isolate a single organism from the uncontaminated soil microcosms that could degrade TDG. A consortium of three bacteria, *Rhizobium* sp, *Achromobacter* sp, and *Stenotrophomonas* sp was needed to biodegrade TDG (Figure 14).

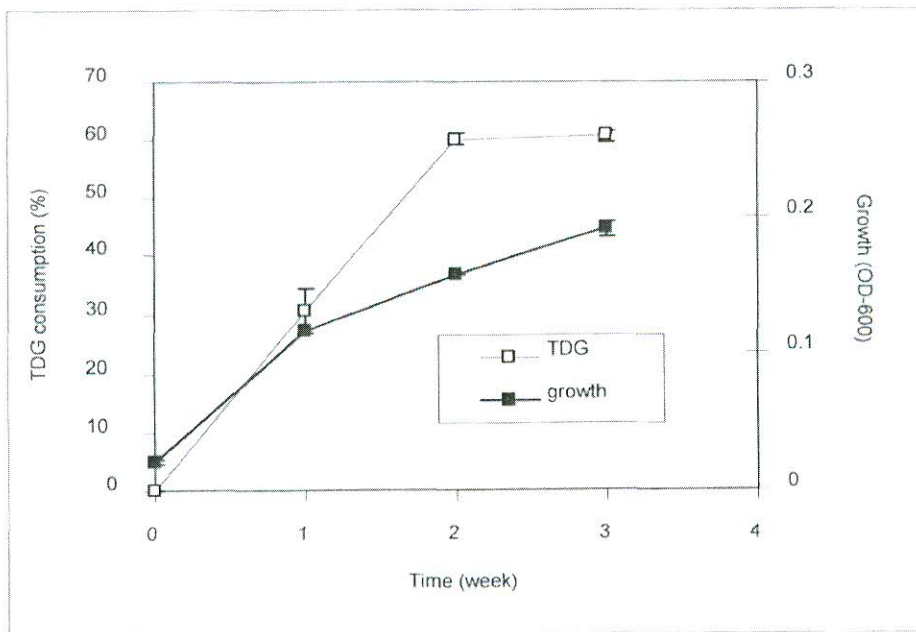


Figure 14 - TDG degradation by a consortium of three bacteria, *Rhizobium* sp, *Achromobacter* sp, and *Stenotrophomonas* sp isolated from clean soil

### 5.5 Constraints to the bio-degradation of 1,4-dithiane and 1,4-thioxane

The experimental work recorded so far has concentrated on the biodegradation of the principal mustard hydrolysis product, TDG. Other mustard breakdown products are also present in the contaminated soil and are characterized by the malodorous compounds 1,4-dithiane and 1,4-thioxane. Both of these compounds were detected at high concentrations at the contaminated site. Based on a limited amount of data, the concentrations of these two chemicals in sampling exercises A and B indicates a correlation, however this cannot be demonstrated for sampling exercise C (Figures 15(a) – (c)). A correlation is not unexpected because the two compounds are very similar chemically, and are probably produced by very similar processes. However, attempts to isolate their degradation products were not successful. Similarly, attempts to isolate dithiane and thioxane degrading microorganisms also failed suggesting that

<sup>1</sup> OD600 - A method of estimating the number of organisms in a culture based on the Optical Density at a wavelength of 600nm.

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microbiological constraints, such as low solubility in water, and hence low bioavailability, play an important part in the fate of these compounds in soil.

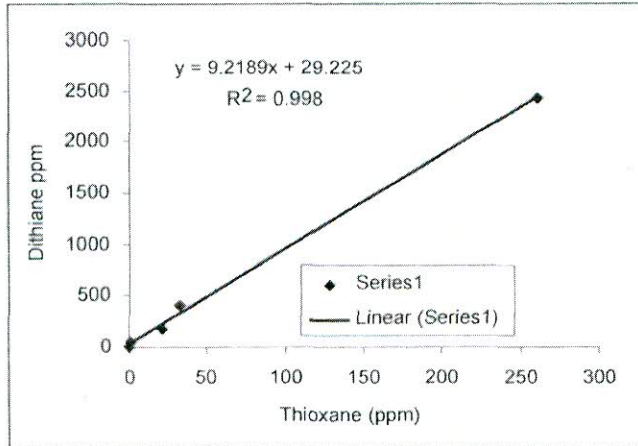


Figure 15 (a) - Relationship of concentrations between 1,4 dithiane and 1,4 thioxane in soils contaminated by mustard and collected during Exercise A.

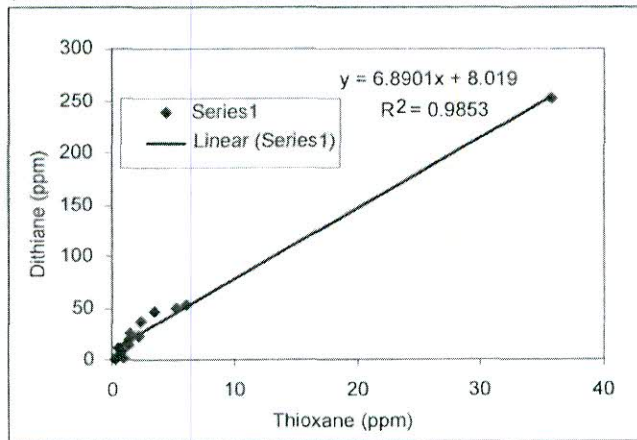


Figure 15 (b) - Relationship of concentrations between 1,4 dithiane and 1,4 thioxane in soils contaminated by mustard and collected during Exercise B

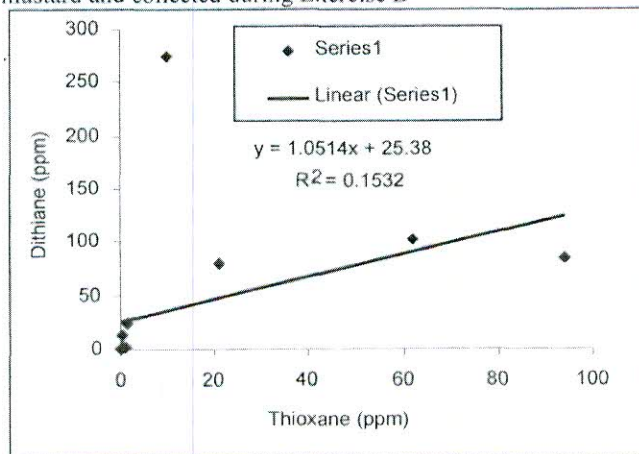


Figure 15 (c) - Relationship of concentrations between 1,4 dithiane and 1,4 thioxane in soils contaminated by mustard and collected during Exercise C.

## 6 Discussion

In contaminated soils, higher mustard concentrations led to lowering of pH with a negative linear correlation ( $r = -0.933$ ,  $p = 0.020$ ) between pH 8.5 and 4.5 and up to 19 ppm of mustard (Figure 1 and 2). This effect, in turn, inhibited biodegradation rates in the contaminated sites. Biodegradation rates rapidly increased after a pH of about 5.5 and all of the TDG was consumed after two days once the pH was over 6.5. The optimal pH for biodegradation of the mustard hydrolysis product, thiodiglycol (TDG), in microcosms of soil free from mustard, was pH 8.5 (Figure 3), although any pH above ~6 gave rise to significantly enhanced rates of degradation.

The effect of mustard contamination on pH may also serve as a useful diagnostic tool when assessing the extent of mustard contamination across a site. The presumption being that areas of higher mustard contamination would have a lower pH than those that were less contaminated.

Significantly higher sulphate and chloride concentrations were also detected in contaminated sites (Figure 5), presumably due to the mineralization of the mustard to  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ , chloride and sulphate. Using isotopic techniques, it may be possible to assess the extent of biodegradation of mustard in situ by measuring the isotopic ratios of sulphate and chloride against naturally occurring ratios. This relatively simple analysis could enable a forensic diagnostic approach to understanding the history and predicting the future degradation of mustard within contaminated sites.

Aeration gave rise to some enhancement in biodegradation of TDG in mustard contaminated soil from the field (Figure 8 and 9) and addition of nitrate also enhanced degradation (Figure 7). Controlling the pH, oxygen and nitrate balances concentrations to their optimal conditions on contaminated sites may overcome existing constraints to bioremediation and accelerate the naturally occurring processes.

Acidic pH, lack of adequate aeration of the soil due to its clayey nature, and the high water table at the site tested are likely to have constrained the biodegradation rates and contributed to the accumulation of TDG in soils during the 60 years since contamination occurred. The persistence of TDG may also be attributed to the formation of complex aggregates with mustard and intermediate hydrolysis products. It is these aggregates that are suspected of being the source of the TDG measured in soil and ground water. In addition, lower nitrate concentrations (Figure 6) in contaminated soils indicate that limitation of this nutrient and electron acceptor may further limit biodegradation. Initial TDG degradation intermediate analysis indicates that the breakdown of TDG follows an oxidation pathway, via a mono- followed by a di-acid prior to complete mineralisation as sulphate, water and carbon dioxide.

The present work indicates that minor intervention at a mustard contaminated site should lead to accelerated destruction of TDG and, possibly, of the residual mustard. Experimental trials should be carried out on bioremediation of mustard contaminated soils in the field to prove the concept. It is proposed that the contaminated soil should be aerated (rotovation) and limed with the addition of a high nitrogen fertiliser in an attempt



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to remove the constraints to microbial activity and kick-start the naturally occurring processes. Furthermore, cultivation of the soil should help to break-up the mustard TDG aggregates and allow hydrolysis to continue while the rapid removal of the TDG by biodegradation should drive the hydrolysis equilibrium to the right allowing the reaction to take place more rapidly.

Ideally all of the methods discussed should be used in conjunction with each other, however, it should be noted that there may be other inherent risks associated with intrusive work that may require the use of further engineering controls (e.g. a vapour containment suite). It may be more expedient to use the methods that minimise intervention (eg lime and nitrogen fertiliser) to kick-start the biodegradation process prior to attempting intrusive work.

TDG alone has no significant impact on the soil microbial Community Level Physiological Profile (CLPP) and the soil microbial community (Figure 9 and 10). However, the results of tests with contaminated soil show that the microbial CLPP was lower in the contaminated soils (Figure 11), especially the heavily contaminated soil which showed far fewer diverse microbial functional groups, compared to the uncontaminated soil. This means that the microbial community in the contaminated soil was impoverished compared to the large number of species in the clean soil control sample. The reduced range of bacterial species present in the contaminated soil suggests that the microbial community is under pressure due to either the toxic nature of the xenobiotic chemicals in the soil or because of a lack of nutrients, or both.

Experiments were therefore carried out in an attempt to isolate novel bacterial strains capable of degrading mustard from the contaminated soil. One strain, identified by 16S rRNA gene sequencing as a *Burkholderia* sp., was shown to be capable of utilising TDG as a sole carbon source for growth (Figure 12). It was also demonstrated that this species could grow on mustard hydrolysate, derived from the contents of old chemical munitions, which it utilised as a sole carbon source (Figure 13). This is the third bacterial strain (following *Pseudomonas pickettii* and *Alcaligenes xylosoxidans*[2]), identified to date, which can utilise TDG as a sole carbon source.

To test whether bacteria in all soils are capable of biodegrading TDG, three very different soil types were taken from across the UK and approximately 1000 ppm TDG was added to each in a microcosm. In all cases, biodegradation occurred and this study strongly suggests that the ability of soil microbes to degrade TDG is a common and widely distributed trait. It is significant that it was not possible to isolate a single organism with these capabilities from uncontaminated soil where a consortium of three bacteria, *Rhizobium* sp, *Achromobacter* sp, and *Stenotrophomonas* sp was needed to biodegrade TDG. This interesting contrast is good evidence that evolution of TDG-degrading capability has occurred in the contaminated soils. This observation also confirms that the proposed bioremediation strategy, of optimizing conditions for this specialist strain to degrade mustard hydrolysis products on site, would be effective. Therefore, providing hydrolysis of mustard occurs, further biodegradation is likely (i.e. the constraints to biodegradation may relate more to hydrolysis and chemical reactions than microbial capabilities). However, in samples taken from different locations on the contaminated site a significant quantity of TDG and TDGA were often detected (Figure 4). At these

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locations, the constraints must relate to both a defective activity of both microorganisms and chemical hydrolysis.

Further investigation of the genetics and physiology of the TDG degrading strain isolated from the contaminated site will be useful to develop bioremediation in strategies in the future. An understanding of the environmental fate of 1,4-dithiane and 1,4-thioxane will be important in this work as these two malodorous mustard degradation products, or manufacturing by-products (which are often present in mustard contaminated soil) would also need to be removed.

## 7 Conclusions

- 7.1 In contaminated soils, higher mustard concentrations led to lower pH which inhibited contaminant biodegradation rates. The optimal pH for biodegradation of TDG in uncontaminated soils was shown to be 8.5 although pH values above ~6 gave rise to significantly enhanced degradation rates. Significantly higher sulphate and chloride concentrations were detected in contaminated soil due to the mineralisation of mustard. The low oxygen and nitrogen levels observed in the contaminated soil were shown to be inhibiting biodegradation.
- 7.2 The measurement of soil pH may provide a good indication of the extent of mustard contamination across a site.
- 7.3 Although TDG alone did not impact on the soil microbial community, in contaminated soils these communities were impoverished compared to the large number of species in the clean soil control sample.
- 7.4 It was not possible to isolate a single organism capable of degrading TDG from uncontaminated soil where a consortium of three bacteria, *Rhizobium* sp, *Achromobacter* sp, and *Stenotrophomonas* sp are needed.
- 7.5 One bacterial strain, identified as a *Burkholderia* sp., was isolated from contaminated soil and shown to be capable of utilising TDG as a sole carbon source for growth. This is good evidence that evolution of TDG-degrading capability has occurred in the contaminated soils.
- 7.6 Minor intervention, by rotovation to aerate and incorporate lime and a high nitrogen fertilizer, at a mustard contaminated site should lead to accelerated destruction of TDG and, possibly, of the residual mustard (of these liming is anticipated to have the greatest impact).

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- 7.7 Attempts to isolate the degradation products of 1,4 dithiane and 1,4 thioxane were not successful. Similarly, attempts to isolate degrading microorganisms for these compounds also failed, suggesting that constraints, such as low solubility in water, and hence low bioavailability, play an important part in the fate of these compounds in soil.

## 8 Recommendations

- 8.1 Trials should be carried out on bioremediation of mustard contaminated soils by incorporating lime and a high nitrogen fertiliser in conjunction with the aeration (rotovation) to remove the constraints to microbial activity and kick-start the naturally occurring processes.
- 8.2 Soil pH should be recorded during future site investigations so that its use as a diagnostic tool to assess the extent of mustard contamination can be evaluated.
- 8.3 Further investigation of the genetics and physiology of the TDG degrading strain isolated from the contaminated site should be carried out to provide a better assessment of its potential as an effective agent for remediation.
- 8.4 Work to improve understanding of the environmental fate of dithiane and thioxane should be carried out with a view to devising a strategy for their removal from contaminated soil.

## 9 Acknowledgments

- 9.1 The project manager for this research was [REDACTED] Environmental Knowledge Transfer Network in the Department of Earth Sciences, University of Oxford. Microbiology was led by [REDACTED] of the Natural Environment Research Council Centre for Ecology and Hydrology in Oxford, with the lead scientist being [REDACTED].
- 9.2 [REDACTED] and [REDACTED] of the University of Nottingham provided expertise on site characterisation and risk analysis. The project was funded by the Natural Environment Research Council and the Engineering and Physical Sciences Research Council.
- 9.3 [REDACTED] formally of Dstl, arranged the supply of contaminated soil samples and the brought together the research findings to complete this report. Funding for this report was provided by the Ministry of Defence Scientific Risk Management team.

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